

Review Article

Phage therapy: awakening a sleeping giant

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For a century, bacterial viruses called bacteriophages have been exploited as natural antibacterial agents. However, their medicinal potential has not yet been exploited due to readily available and effective antibiotics. After years of extensive use, both properly and improperly, antibiotic-resistant bacteria are becoming more prominent and represent a worldwide public health threat. Most importantly, new antibiotics are not progressing at the same rate as the emergence of resistance. The therapeutic modality of bacteriophages, called phage therapy, offers a clinical option to combat bacteria associated with diseases. Here, we discuss traditional phage therapy approaches, as well as how synthetic biology has allowed for the creation of designer phages for new clinical applications. To implement these technologies, several key aspects and challenges still need to be addressed, such as narrow spectrum, safety, and bacterial resistance. We will summarize our current understanding of how phage treatment elicits mammalian host immune responses, as well bacterial phage resistance development, and the potential impact each will have on phage therapy effectiveness. We conclude by discussing the need for a paradigm shift on how phage therapy strategies are developed.

Introduction

Bacterial infectious diseases are one of the biggest threats to health and food security worldwide [1–3]. Each year, an estimated 2.5 million people acquire antibiotic-resistant infections that claim at least 50 000 lives across Europe and the USA alone [3]. Most importantly, the drug pipeline to combat these emerging threats has become scarce [3,4]. Thus, it is vital that new antibacterial agents and strategies are developed to meet the growing demand for antibacterials [3,5].

Long before the discovery of antibiotics, bacterial viruses called bacteriophages (phages) were used to treat and prevent infectious disease in humans and animals [6–8]. In the early years, phage therapies resulted in mixed success, in large part due to a poor understanding of the viruses themselves, as well how they infect and kill bacteria [6,7]. With the discovery of penicillin, phage therapies were largely superseded with the dawn of the antibiotic era. Now, with the threat of MDR (multi-drug resistant) infections, there has been a refocusing on the therapeutic use of phages to provide a new solution to eradicate unwanted bacteria, such as those associated with infectious diseases and gastrointestinal microbe dysbiosis [9].

For the past three decades, a considerable amount of knowledge has been gained with regard to the genetic, structural, functional, and ecological features of phages, for examples [10–12]. Accordingly, the progression in the development of phage therapies has grown steadily across a wide variety of diseases (Table 1). Recently, advances in genome engineering have led to the design of semisynthetic and synthetic phages with specialized antibacterial features, drastically broadening the breadth of phage therapies [13]. This review provides a general overview of phage therapy, both natural and synthetic-based, and summarizes the current progress in defining the ability of phages to provoke mammalian host immune responses and bacterial resistance. We also discuss new perspectives that should be satisfied for phage therapies to become more effective in treating infectious diseases.

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Modalities of phage therapy

The purpose of phage therapy is to eradicate unwanted bacteria. This can be accomplished with two differing strategies, using either natural phages (Figure 1) or engineered (i.e. synthetic-based) phages (Figure 2). Both technologies provide multiple modalities that together can cover a broad range of applications in the treatment and prevention of infectious diseases (Table 1).

Natural phage therapies

It has been fairly standard practice to use viruses as antibacterial agents that fall into the order Caudovirales, which are double-stranded DNA viruses with a tail appendage [14]. The main advantage of using tailed phages is their ease of discovery as they are naturally found everywhere bacteria exists, including in the environment, and even inside plants, animals, and humans [15]. Another major advantage is their inherent combined bacteriostatic and bacteriolytic mode of action (Figure 1). Tailed phages infect bacteria and hijack several cellular systems to produce progeny virions before being released by rupturing the cell. This can all happen within minutes after initial contact [11]. By differing in mode of action to static antibiotic drugs, most therapeutic phages are not only effective against antibiotic-sensitive and antibiotic-resistant bacteria, when co-applied they are often a synergistic therapy [16]. Further differentiating from static drugs, phages can indefinitely repeat killing cycles while amplifying in numbers as long as susceptible bacteria are present, thus providing a density-dependent dose at the site of infection.

Another inherent feature of phages is that each has its own specificity toward bacteria, with some infecting only a few strains within a species, while others can infect many strains even across different related species [17]. This relatively narrow spectrum of individual phages can be assimilated into a tool that can very precisely remove only unwanted bacteria, in contrast with the blind action of antibiotics that kill many bacteria. The adverse effects of broad-spectrum antimicrobials have been long-known. For instance, antibiotics most often rapidly alter gastrointestinal microbiota composition, referred to as dysbiosis, with potential immediate effects on health [18]. These include diarrhea and selection of MDR opportunistic pathogens that can cause acute infections. In the long term, dysbiosis can indirectly affect health by removing mutualistic microbes that provide beneficial physiological processes for the host and can participate in host immune homeostasis [18]. The precision of phage antibacterial activities can help maintain healthy gastrointestinal eubiosis [19,20].

Broad-spectrum antibiotics do allow for the treatment of undiagnosed causative agents with some certainty of success [1]. Conversely, even phages with the broadest bacterial spectrums still do not come close to those of broad-spectrum antimicrobials. However, phage narrow host ranges cannot be assumed to exist in nature [17]. For instance, nearly all therapeutic phages have been isolated and continually propagated using a single

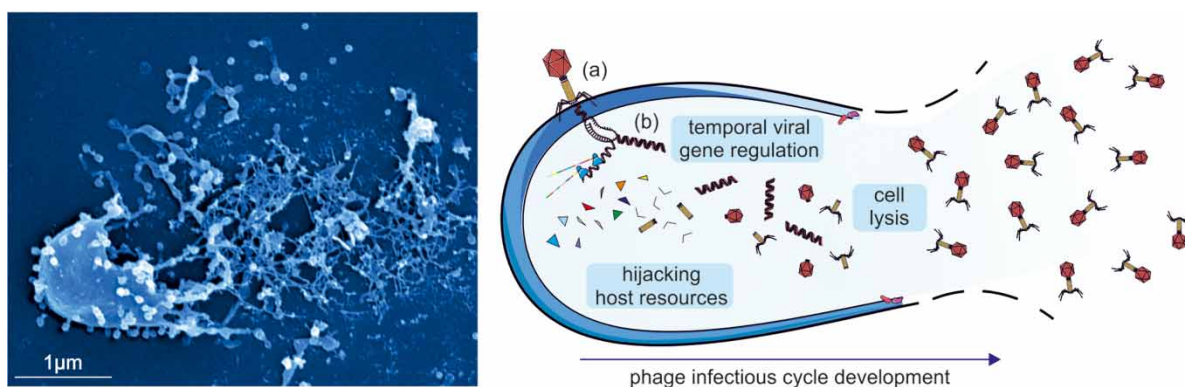


Figure 1. The bacteriolytic lifecycle of phages.

(Left) Scanning electron microscopy of a bacterial cell (*Acinetobacter baumannii*, false color) being lysed by phage (vB-GEC_Ab-M-G7) [93] infection. (Right) Phages infect bacteria by first attaching (a) to susceptible cells via specific surface receptors before injecting (b) its viral genome into the cytoplasm. Then, the viral genome hijacks the bacterium whereupon progeny virions are synthesized and assembled. Most phages employ a viral-encoded cell lysis system where holins perforate and weaken the cytoplasmic membrane and endolysins degrade the cell wall peptidoglycan, which then causes the bacterium to violently rupture. Cell lysis can occur within minutes to hours depending on each phage and metabolic status of the bacterium.

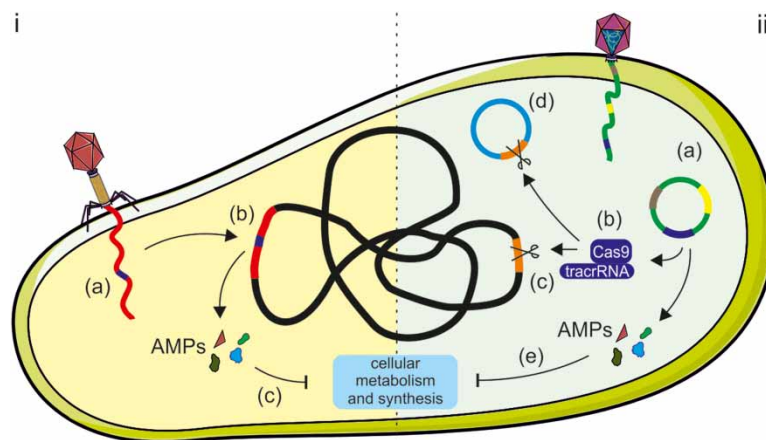


Figure 2. Overview of engineered nonlytic antibacterial phage technologies.

(i) Temperate phage engineered to deliver synthetic gene network (blue) (a), undergo a latent lifecycle after infection, called lysogeny. Here, the viral genome (red) integrates into the bacterium's chromosome as a prophage (b) where it can express antimicrobial proteins (AMPs) that interfere with intracellular processes and cause bacterial death (c). (ii) Phagemids can also deliver synthetic gene network(s) (blue) on a synthetic plasmid (a) that encode for antibacterial proteins, such encoding a RNA-guided CRISPR-associated (Cas) nucleases (b) for sequence-specific (orange) nonlytic bacterial death (c) and plasmid removal (d). Phagemid plasmids can also encode for AMPs (e).

Table 1 Brief compilation of phage therapy experimental and clinical studies

We refer readers to each reference for phage type(s) and therapy outcome.

Disease	Causative species	Animal	Human
Diarrhea	<i>Escherichia coli</i>		[20,52]
	<i>Shigella sonnei</i>	[94]	
Endocarditis	<i>Pseudomonas aeruginosa</i>	[16]	
Meningitis	<i>E. coli</i>	[95]	
Osteomyelitis	<i>Staphylococcus aureus</i>	[96]	
Otitis	<i>P. aeruginosa</i>	[97]	[66]
	<i>E. coli</i>	[33,35,38,98]*	
Peritonitis	<i>S. aureus</i>	[99]*	
	<i>P. aeruginosa</i>	[100]	
	<i>E. coli</i>	[101,102]	
Respiratory infections	<i>P. aeruginosa</i>	[101,102]	
	<i>E. coli</i>	[103]	
	<i>Klebsiella pneumoniae</i>	[104]	
Skin infections	<i>S. aureus</i>	[39]*, [53]	
	<i>K. pneumoniae</i>	[105]	
Venous leg ulcers	<i>P. aeruginosa</i> , <i>S. aureus</i> and <i>E. coli</i>		[65]

*Synthetic phage.

pathogenic strain. Moreover, selection priority is largely given to those phages that rapidly kill this strain, before screening on an empirical collection of strains *in vitro* to determine host ranges. Modifying isolation procedures and growth conditions can favor the isolation of phages with much broader host ranges [17]. For example, phage Mu is polyvalent, making it able to infect species of *Escherichia*, *Citrobacter*, *Shigella*, *Enterobacter*, and *Erwinia* [17]. Moreover, over 700 strains of *Staphylococcus aureus* can be lysed by a single phage [21]. Formulations of phage mixtures (cocktails) may also provide a solution to achieve mid-spectrum

phage therapies. Thus far, there are few guidelines for inclusion of phages in cocktails. Most often formulations are empirical based on each phages host range *in vitro*. This often leads to formulations requiring a large number of phages to provide a theoretically sufficient bacterial spectrum. However, mixing several phages may significantly complicate GMP (good manufacturing practices) production, while also increasing development cost and safety regulations. Moreover, the simplistic view that an ideal phage *in vitro* should work as well *in vivo* is biased by experimental models where optimal conditions are tuned in contrast with clinical situations. Further study is needed for cocktail formulation, from devising proper isolation procedures and growth conditions for therapeutic phages to accounting for the inherent ability of phages to evolve and kill once non-susceptible bacteria [22–24]. How this can be exploited and controlled for the design of phage cocktails is still a pending question.

Synthetic phage therapies

Current antibiotic therapies are the result of decades of iterative implementations coupled to experimental and clinical studies to deliver highly effective treatment dependent on antibiotic type and setting. One major breakthrough was the use of synthetic approaches to expand the natural narrow spectrums of small molecule antibiotics, such as engineer approaches to bypass or break the permeability of Gram-negative bacteria [25]. Recent advances in sequencing technologies and genetic engineering have allowed for the design of phages with more predictable and domesticated therapeutic properties. For instance, recombinant phages can be created to have hybrid tail fibers from related phages to broaden bacterial host ranges [26,27]. Moreover, added genes can improve phage diffusion by expressing exopolysaccharide-degrading enzymes specific to target biofilm [26]. Phage capsid surfaces can be decorated with cell-penetrating peptides (a technique called phage display) to enter mammalian cells and kill intracellularly residing bacteria [28,29].

Natural phage therapies rely on causing bacterial death through rupturing cells [30]. However, rapid bacterial lysis might result in the release of endotoxin and inflammatory mediators into the surrounding environment with adverse effects. In contrast, phages can be engineered to be bacteriostatic by deleting genes responsible for lysis (e.g. endolysin) [31–33]. Some tailed phages, referred to as ‘temperate’, can undergo a latent lifecycle after infection, called lysogeny, and deliver synthetic gene networks with desirable nonlytic antimicrobial properties (Figure 2). Here, the viral genome either integrates into the bacterium’s chromosome as a prophage or remains free in the cytoplasm, which is then replicated alongside the bacterium until conditions favor reactivation to produce virions. Although temperate phages are generally avoided in natural phage therapies, they have been used to deliver synthetic gene networks that can disrupt cell–cell communication between bacteria involved in biofilm formation [34], or to work as adjuvants to antibiotics, such as by repressing DNA repair mechanisms [35] or overexpressing sensitizing proteins [36]. The drawback of these approaches is that the temperate phages would be inherently nonlethal, which one can argue would unnecessarily complicate treatment over using bacteriolytic phages. Prophage-encoded genes also carry the risk of providing a variety of benefits for their bacterial hosts. These beneficial genes are often contained within ‘moron’ elements and suggest that temperate phages have a symbiotic relationship with bacteria rather than being purely parasites as virulent (lytic) phages [37].

Phages can be designed to carry plasmids of known genetic content containing a phage origin of replication that can be packaged into replication-incompetent phage particles using a prophage helper system, which is referred to as a phagemid. A plasmid can be designed to encode antimicrobial peptides and toxin proteins that can be delivered by a phagemid to cause nonlytic bacterial death [38]. Instead of directly killing bacteria, phagemids can be used to enrich, for example with antibiotic-sensitized populations, by delivering a sequence-specific CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system [13,39,40]. CRISPR RNAs can be programmed to target antibiotic-resistant genes on bacterial born plasmids, which when delivered by phagemids can reverse antibiotic resistance and eliminate the transfer of resistance between strains [39–41]. In the case of the genomically encoded targeted sequences in a single virulent bacterial species, or even an antibiotic-resistant gene, CRISPR phagemids can cause unrepairable chromosome damage in only targeted bacteria [39,42]. Most bacteria have poor non-homologous end-repair systems, so a CRISPR-induced double-stranded break in the genome can be lethal. Although phagemid therapies may provide a marked benefit over therapies using lytic and temperate phages, as phagemids lack replication, it will likely translate into requiring much higher doses compared with self-amplifying natural phages [39].

Immunological complications with phage therapy

Phage therapy encompasses several modalities, but common to all is the use of a virus that can present immunological complications for the host. After a century of study, however, there have been no reports of phage particles causing diseases in humans, animals, and plants, despite being ubiquitous in food, intestinal microbiomes, and the environment [43]. This would imply that phages are safe and generally well tolerated by mammals. Nonetheless, it still remains a major concern that phages given as therapeutics will either adversely affect the patient or be neutralized by the host immune defenses.

Mammalian host defenses consist of innate immunity that acts as the first line of fast-acting defenses against invaders, which remain active until the second line of long-lasting adaptive (specific) immunity develops. Of these two arms, adaptive immunizations toward phages have long been described [44–46]. In fact, use of the ‘filamentous’ phage Φ X174 has been described since the 1960s as a method to assess specific antibody responses in patients with immunodeficiencies [47]. Low numbers of pre-existing serum antiphage antibodies toward tailed phages have also been demonstrated [46,48,49], which is not surprising with the high frequency of natural contact that humans have with tailed phages [43]. It has also been demonstrated that antibodies can be generated toward phages given therapeutically to treat bacterial infection in human [49] and animal [48,50]. Antiphage antibodies have been found to have an affinity for specific viral structural components, for instance, against the non-essential highly immunogenic outer capsid (Hoc) protein of T4-like phages [48–50]. In contrast, several studies have shown that other tailed phages have been unable to stimulate antibody production [20,51,52] or T-cell cytokine responses [53]. However, the level of anti-Hoc antibody responses has been found to be largely influenced by the route of phage administration (e.g. oral versus intraparietal) and time, taking several weeks of high doses before being produced in mice [49]. Moreover, phages with Hoc protein analogs are extremely rare in nature [48].

Antiphage antibodies, whether pre-existing or developed during treatment, appear not to be a limiting factor for successful phage therapy [49]. Sulakvelidze et al. [19] also postulated that antibody responses would not be a significant problem during the treatment of acute infection because phage activity can reduce bacterial burden more rapidly than the host can produce specific antibodies, which take days to weeks. On the other hand, generated antiphage antibodies could prevent the reuse of certain phages, as pre-immunization with purified Hoc protein can reduce the therapeutic efficacy of phage T4 in mice [48]. To remedy this, technologies can be applied to limit antiphage antibody neutralization, such as covalent binding (conjugation) of non-immunogenic polyethylene glycol molecules to phage capsid proteins [54] or entrapment of phages in liposomes [55] to evade viral immune sensing.

Host innate immune response toward phages is far from being defined. Innate immune cells sense microbes through specialized pattern recognition receptors (PRRs) on their cell surface (e.g. Toll-like receptors) [56]. Unlike their bacterial counterparts, phages are incapable of stimulating most PRRs due to their much simpler biochemical makeup, only having proteomic structures surrounding genetic material. Nonetheless, certain phages are rapidly cleared from the blood and liver by phagocytes [57,58] or are readily phagocytized by dendritic cells *in vitro* [59]. In humans, patients who received several weeks of phage therapy for staphylococcal infections, including rhinitis, sinusitis, chronic bronchitis and urinary tract infections, experienced slightly fewer circulating mature neutrophils with a corresponding increase in neutrophil precursors [60]. This suggests that bacterial products released after phage killing are eliciting proinflammatory cytokine responses leading to further recruitment of immature neutrophils [60]. Phage preparations have also been shown to modulate immune cell function, such as reduce reactive oxygen species production by phagocytes [61], or promote dendritic cell maturation and IL-12p40 cytokine production [62]. Although, phagocyte functions appear not to be adversely affected by phage therapy [63], the mechanisms by which immune cells sense phages remains unknown. It cannot be ruled out that the weak immune responses elicited by phages are artifacts of the preparative process in which bacterial debris can contaminate [64]. If and how innate and adaptive immune systems sense and respond to phages in general or, on an individual basis, still needs thorough investigation.

Tailed phages have so far been very weak- or non-elicitors of host immune responses, which could explain why all clinical trials have all reported tailed phages were well tolerated by patients [20,52,65,66]. Recently, Smith et al. [47] conducted a retrospective study of the systemic administration of phage Φ X174, a known elicitor of humoral responses, after patients had received primary, secondary, and tertiary phage immunizations. The study found that no adverse effects were reported by most patients, with only a few reporting mild effects such as rash, headache, and fever [47]. This suggests that phage treatments should be safe in the majority of settings, but further study is needed to outline side effects across all clinical settings.

Phage resistance

Phage therapies are not without the risk of bacteria acquiring resistance [67,68]. Phage resistance most commonly develops through the down-regulation, shielding, or modification of bacterial cell surface receptors required for viral attachment [69,70]. To ensure survival, evolved phages tend to bind to highly conserved surface receptors, such as those required for virulence [71–73]. Bacteria can also evolve or be acquired through lateral transfer, a diverse array of antiviral so-called immune mechanisms. These include forms of innate restriction–modification, toxin–antitoxin, and altruistic bacterial abortive infection defenses, as well as CRISPR–Cas systems that serve as a form of adaptive immunity [67]. Despite our growing understanding of phage resistance mechanisms, it is too early to accurately predict their impact on phage therapy.

Phage resistance is expected to not have sustainably negative effects as seen with antibiotic resistance. Phages outnumber bacteria 10-fold with enormous diversity [15]. Therefore, new effective phages are principally unlimited against most pathogenic bacteria [3,74]. In addition, phage resistance is not always detrimental to treatment, because in many cases acquired resistance is costly for the bacterium (e.g. loss of virulence toward their hosts, lower growth rate, decreased lifespan, or loss of the ability to attach or invade to mammalian cells) [70,71,73,75]. In addition, acquiring phage resistance can cause bacteria to become more antibiotic-sensitive [76,77]. However, there are examples where no observable cost to the bacterium was associated with gained phage resistance [78]. Importantly, antiphage immunity has not been implicated in antibiotic resistance [69]. Phages can evolve or acquire counter-systems to circumvent bacterial immunity [79]. For example, some phages have produced their own methyltransferases to defend against bacterial restriction–modification systems [80], whereas other phages encode for anti-CRISPR–Cas systems [79,81].

Like with antibiotic therapy, bacteria ‘persisters’ within a predominately susceptible population can exhibit ‘phenotypic tolerance’ to phages without acquiring new mutations or immunity systems [68]. Although not well understood, this very small fraction of non- or slow-growing persisters emerges stochastically in response to environmental queues or from errors in metabolism [82]. They are genetically identical with susceptible cells but evade phage attack, which when phages are cleared from the host may revert to a growing state causing post-treatment relapses [68,83]. Although phenotypic tolerance is non-inheritable, it may provide a time window for otherwise susceptible bacteria to acquire genetic resistance. It is anticipated that periodic dosing and timing control is intrinsic with the proper therapy design, which could be determined by the dynamics of persisters.

Clinical status of phage therapies

Phage therapy is currently not used medicinally in the Western world; apart from Poland and the Eastern countries Georgia and Russia, which have continued to use phages when antibiotic therapies proved ineffective [7,8,19]. Although treatment protocols in these countries were not developed among western standard clinical studies, their practical use does provide some guidance for future clinical development.

Only a few human clinical trials have evaluated therapy using natural phages. Completed phase I trials on treating chronic venous leg ulcers caused by *Pseudomonas aeruginosa*, *S. aureus*, or *Escherichia coli* [65] and chronic rhinosinusitis caused by *S. aureus* (ampliphbio.com) indicated that overall phages were well tolerated. All patients in the latter study experienced either a reduction or complete eradication of *S. aureus* infection in their sinuses. Similarly, a phase I/II trial on treating chronic otitis caused by *P. aeruginosa* with phages also reported no adverse effects and that bacterial burden was significantly lower in treated patients [66]. In contrast, a recently completed phase II trial using phages to treat acute diarrhea caused by *E. coli* in children found no improvement over current standards of care by oral rehydration/zinc treatment [20]. However, *E. coli* numbers were found to be low and mixed infections were found to be frequent, which raised the questions on the causative agent of diarrhea that may explain treatment failure [20]. The first large-scale European Union-funded randomized phase I/II single-blind trial is currently being conducted in 11 hospitals in France, Switzerland, and Belgium, using phage therapy to treat burn wounds infected with *P. aeruginosa* (phagoburn.eu); results are expected mid-2017.

Compared with antibiotics, which development by the pharmaceutical industry follows a clear regulatory path, there is currently no phage products that have satisfied the current regulatory framework [84]. This issue has been acknowledged by the European Medicine Agency, calling for more clinical trials before initiating any adaptation of the regulatory framework [85,86]. Development cost is also another topic that differentiates antibiotics to phages. Isolation of phage is rather straightforward [87] and needs only standard microbiology technologies. Production costs are difficult to anticipate as they are strongly linked to the regulatory path toward which phage products will make their way to patients, and might not be as affordable as expected from laboratory settings.

Conclusion

Phage therapy has several modalities that when combined could have a high impact on infectious disease treatment as an alternative to antibiotics [5,88]. The overall potential has been predicated on access to POC (point-of-care) diagnostics and operational capacity to devise phage cocktails, given that phages may only provide narrow-spectrum therapies [88]. However, the emphasis on care is currently shifting from curative medicine to where practices and interventions are tailored to the individual patient needs. Accordingly, POC diagnostics will likely become readily available to allow for more precise and routine therapies with narrower spectrum, whether with phages or drugs, that minimizes adverse health effects [89]. Then, the use of a phage cocktail, which has been unanimously implemented as the preferred strategy for dealing with the narrow host range of phages, may change toward a more tailored approach [90]. Indeed, some individual phages can provide mid-spectrum coverage depending on the target pathogen (e.g. *S. aureus*). On the other hand, it is unclear if broad-spectrum therapies are always necessary, as some diseases are linked to small sub-group of strains (e.g. *Shigella*) [8]. There is also a growing interest to manipulate the gut microbiota for preventative and therapeutic purpose for which a specific set of phages could represent an ultimate precision solution [9].

Nevertheless, the right path to bring effective and reliable phage therapies to patients remains to be elucidated. We have highlighted three main areas of improving phage selection protocols and criteria, defining mammalian immune responses toward phages, and defining the impact of resistance, which are currently limiting the development of phage therapies. Synthetic solutions, as well as POC diagnostics, will also help narrow choices. We should substantially increase basic research and clinical trials to build on knowledge that will bring phage therapy to patients. Conducting clinical trials, however, with the established regulatory paradigms that are intended to evaluate static broad-spectrum drugs, will not be equipped to evaluate clinical complexity of employing replicative phages for treating infectious disease [86,91,92]. Thus, future experimental and clinical studies will require multi-dimensional outcome profiling to advance toward using phages for more precision medicine tailored to the specific disease and status of the patient [86].

Summary

- Natural- and synthetic-based phage therapies can be used to prevent a wide variety of bacterial diseases in humans and animals.
- Host immune response to phages and risk of bacterial resistance during treatment must be considered but are of minimal risk to phage therapy.
- Human clinical trials are ongoing.
- Using a multi-component integrated strategy will help phage therapies to be more effective in the future.

Abbreviations

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Hoc, highly immunogenic outer capsid; POC, point-of-care; PRRs, pattern recognition receptors.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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